examples related to these implementations are not meant to be limiting. In other implementations, other types of detectors are used such that images of the target objects are not created, but rather only centroids are computed that are indicative of the position of the one or more target objects in the flow cell cavity 268.

As in the un-polarized implementations of the imaging system 100, it is important to control the amount of light in each beam path in order to result in images of approximately the same intensity level at the detector. In addition to the methods previously discussed for light control, in the polarized implementations, the light intensity in the defocus optical path 138 can also be controlled by the angular orientation of the optical retardation plate 212. As the optical retardation plate is rotated the plane of linear polarization also rotates. This results in the second polarization state vector 214 at the polarization beam splitter 208 to be rotated with respect to the plane of incidence so that polarization beamsplitter optical coating 116 splits the incident light into its vector component s- and p- polarization states. Since the p-polarized light is transmitted through the polarization beamsplitter optical coating 116 while the s-polarized light is reflected, the 2R defocused light light 168 is reduced in intensity. The effective beamsplitter ratio at the polarization beam splitter 208 can therefore be varied in this manner. An alternative to the use of neutral density filters in the polarized embodiment is the use of a linear polarizer as a variable transmittance filter. When placed in the linear polarized first transmitted light 136 or the first reflected light 138, the transmittance of the light through the polarizer will vary with the orientation of the polarizer axis.

An active autofocus system 700 is illustrated in Figure 25 to receive from an optical system 702 such as an implementation of the imaging system 100, light 704 by a camera 708. The host computer 720 runs an auto focus system software having a method such as described below. Object light 132 is collected from the target object 108 and image by the optical system 702 onto the camera 708. Light 704 from the optical system is brought to a focus at the camera 708 with the precise focal position under control involving a frame grabber 712, an image processor 716, a host computer 720, a motor driver 724, and a motorized 728. The motorized stage 728 may be configured to move the entire optical

system 700 or any number of optical components of the optical system, such as the camera 708. Alternatively, the motorized stage 728 could be configured to move the target object 102. The host computer 720 controls image acquisition by the camera 708. The frame grabber 712 executes methods for image processing 716, which result in instructions based on one or more autofocus error signals being sent to the motor driver 724 to move the motorized stage 728 the appropriate magnitude and direction so as to maintain objects in focus at the camera.

A method 800 for maintaining objects in focus using the active autofocus system 700 is shown in Figure 26. The method 800 works in conjunction with the optical system 702, such as the imaging system 100 wherein in the imaging system produces imagery such as described with respect to a detector, such as the first detector 120, shown in Figure 16 having two focus areas, such as the 2T1T focus area 224 and the 2R defocused area 232. Imagery is used by the method 800 to produce a focus error signal used to control the position of an adjustable optical component of the autofocus system 700, as described above, to maintain the imagery in focus. The method 800 begins with sample flow being initiated (step 808) and an image being acquired (step 812).

Segmentation processes are used to identify objects of interest (e.g. cells) in the two focus areas (step 816). For these segmented objects, their frequency content is analyzed for each image column (focal plane) (step 820) and compared with each other (step 824) to determine whether the frequency content is balanced, e.g. when the system is in focus. If the frequency content is balanced (YES branch of decision step 828), the system is in focus and no focus correction is required, so the method 800 determines whether additional samples remain and if not (NOT branch of decision step 848) ends. Otherwise (YES branch of decision step 848) goes back to step 812. If the frequencies are not balanced (NO branch of decision step 828), an focus error signal is determined (step 836) (e.g. from the ratio of frequency content) and the required focal shift magnitude and direction is determined (step 840) by reference to a database of stored correction factors or a look-up table. The refocusing optics are then adjusted (step 844) in the proper direction by the required amount and step 848 is executed as described above.

An alternative implementation 900 of the imaging system 100 is illustrated in figure 27 wherein one reflector 904 is used to reflect light in the defocus optical path 122. In this exemplary illustration of the alternative implementation, converging light 902 is received by the amplitude beam splitter 110 and is partially reflected and partially transmitted. The portion of the converging light 902 that is partially reflected is first defocused through the defocus system 126 and then reflected by a reflector 904 on to the amplitude beam splitter 114 to be partially reflected as defocused light 908. The portion of the converging light 902 that is partially transmitted by the amplitude beam splitter 110 is also partially transmitted by the amplitude beam splitter 114 as unaltered light 906.

And exemplary implementation of the imaging system is illustrated in Figure 28 showing the defocus system 126 positioned in the transmission path of the amplitude beam splitter 110. As a result, unaltered light 920 and defocused light 922 have reversed positions compared to other implementations described above. In other implementations using other aspects described above, including but not limited to polarization aspects, dispersion aspects, bi-orientation aspects, and aspects directed to other multiple detector configurations, the defocus system 126 is also position in a transmission path rather than a reflected path.

The numerical aperture, NA, of a microscope objective lens is given by $n*\sin\theta$ where n is the index of refraction of the medium in which the object lies and θ is the half angle of the cone of collected light. The depth of focus of an optical system is the distance through which a detector can be moved along the optical axis forward and backward from focus before the image appears to be out of focus. For a diffraction-limited lens such as a well-corrected microscope objective, Rayleigh's criterion for tolerable defocus allows for $\lambda/4$ wave of wavefront error where λ is the wavelength of the image forming light. This translates to an allowable depth of focus at the image of

$$D' = \lambda/(NA')^2$$

where NA' is the numerical aperture on the image side of the objective. For a system with lateral magnification m, NA' = NA/m and

$$D' = m^{2*}\lambda/(NA)^2$$